ACCELERATED PUBLICATION

BIOTECHNOLOGY and BIOENGINEERING

Total Biosynthesis of Deoxynucleoside Triphosphates Using Deoxynucleoside Monophosphate Kinases for PCR Application

Jie Bao,[1,2] Dewey D.Y. Ryu[1]

[1]Biochemical Engineering Program, University of California, One Shields Ave, Davis, California 95616; telephone: 1-530-752-8954; fax: 1-530-752-3112; e-mail: ddyryu@ucdavis.edu

[2]State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China

Received 19 February 2007; accepted 7 May 2007

Published online 18 May 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21498

ABSTRACT: Polymerase chain reaction (PCR) and other PCR applications for DNA synthesis require deoxynucleoside triphosphates (dNTP) as the essential precursors and substrates. Currently, the dNTP is commercially produced by a chemical method which is environmentally hazardous and costly due to its low yields in both the synthetic reaction and purification processes. In this study, a enzyme technology for the total integrated biosynthesis of all dNTP components is presented. The bioprocess technology developed and reported here involves two sequential enzymatic phosphorylation reactions coupled with the cofactor regeneration starting from deoxynucleoside monophosphates (dNMP) to deoxynucleoside diphosphates (dNDP) in the first reaction step and to dNTP in the second reaction step in the same bioreactor. The four genes encoding these deoxynucleoside monophosphate kinases were cloned into the recombinant *E. coli* and expressed using the recombinant *E. coli* strains. The reaction mechanisms and kinetics of the four kinase enzymes are studied and reported. The total enzymatic syntheses of the four dNTP products were carried out in four separate operations under the high substrate concentrations which emulate the practical application. The optimal process conditions were carefully investigated and complete conversion of dNMP to dNTP at high substrate concentration have been achieved. The purity and quality of dNTP products obtained from this work were analyzed and found to be at least equivalent or better than the commercially available dNTP products. The PCR application of dNTP products obtained from this work were also evaluated for isolating and amplifying genes of different sizes from different organisms. The PCR performance test also showed an equivalent quality as compared to the commercially available dNTP. The bioprocess technology developed and reported here for production of dNTP will provide economically competitive and environmentally friendly viable technology for the industry and research community as compared to the chemical technology currently in use.

Biotechnol. Bioeng. 2007;98: 1–11.

© 2007 Wiley Periodicals, Inc.

KEYWORDS: total biosynthesis; deoxynucleoside triphosphates (dNTP); deoxynucleoside monophosphate kinases (dNMP kinases); PCR application; DNA biosynthesis

Introduction

PCR and other PCR-based applications require dNTP as the essential precursor subunits for DNA synthesis. There are four components of dNTP: deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP). The demand for dNTP is steadily increasing due to the increasing PCR application for DNA biosynthesis by the biotechnology research community and industry (Erlich et al., 1991; Pavlov et al., 2004).

Currently, the dNTP is commercially produced by a chemical method (Chambers and Khorana, 1957; Chambers et al., 1957; Smith and Khorana, 1958). The method involves the reaction of tri-*n*-butylammonium salts of its corresponding dNMP and orthophosphoric acid with dicyclohexylcarbodiimide (DCC) in organic solvents such as, pyridine or dimethylformamide (DMF). The chemical method requires these toxic solvents (EPA Toxics Release Inventory, 2001). The dNTP yield is approximately 40–80%,

Correspondence to: D.D.Y. Ryu

Contract grant sponsor: National Science Foundation of China
Contract grant number: 20676037
Contract grant sponsor: Shanghai Pujiang Program
Contract grant number: 06PJ14022
Contract grant sponsor: Industrial Gift Research Fund of Dewey Ryu at University of California, Davis, CA

© 2007 Wiley Periodicals, Inc.

depending on the specific chemical process used and the different dNTP component. The purification of individual dNTP component from the reaction mixture is complex. It requires separation of unreacted dNMP, dNDP, DCC, and orthophosphoric acid, as well as such byproducts as deoxynucleoside tetra- or penta-phosphates, and the reduced DCC. The separation of dNTP from dNDP is rather difficult and costly because of the similar molecular properties between the intermediates and products. The chromatographic purification step is required for removal of the intermediate dNDP from the dNTP product. In addition, the pyridine or DMF solvents must be recovered, separated, and recycled due to the stringent requirement for waste disposal guidelines. Therefore, the chemical method is environmentally hazardous. It is also costly due to its low yields in both reaction and purification steps.

To overcome these problems involved in the chemical method, such as low dNTP yield and use of toxic solvent, an enzymatic method has been attempted for biosynthesis of dNTP (Ladner and Whitesides, 1985) They described an enzymatic synthesis for dATP, one of the four dNTP components using two commercially available enzymes adenylate kinase (AMP kinase) and pyruvate kinase (PK). However, the dATP yield was only about 60%. A complicated purification procedure similar to the chemical method was still required to remove the unreacted dNMP and the dNDP by-product. Zhang (2003) described an enzymatic method of dNTP using ribonucleoside-triphosphate reductase (EC 1.17.4.2) to remove one hydroxyl group each from the ATP, CTP, GTP or TTP substrates. Similar to Ladner and Whitesides (1985), the conversion was very low and the purification too costly. Furthermore, the enzyme used, ribonucleoside-triphosphate reductase, is an unstable enzyme and not suitable for industrial application.

In this study, we present a strategy for the total integrated biosynthesis of all four dNTP components, dATP, dGTP, dCTP, and dTTP using dNMP as the starting material and four dNMP kinases. The method consists of two sequential enzymatic phosphorylation reactions starting from dNMP to dNDP in the first step, and to dNTP in the second step, as shown in Figure 1. The first phosphorylation reaction step is catalyzed by deoxyadenylate monophosphate kinase (dAMP kinase) for phosphorylation of deoxyadenosine monophosphate (dAMP) to deoxyadenosine diphosphate (dADP). Similarly, deoxyguanylate monophosphate kinase (dGMP kinase) for deoxyguanosine monophosphate (dGMP) to deoxyguanosine diphosphate (dGDP), deoxycytidylate monophosphate kinase (dCMP kinase) for deoxycytidine monophosphate (dCMP) to deoxycytidine diphosphate (dGDP), and deoxythymidylate monophosphate kinase (dTMP kinase) for deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP), respectively. The dATP is used as the phosphate donor and regeneration cofactor in the first reaction step. In the second step, the four dNDP intermediate products are further phosphorylated to produce the four dNTP products by

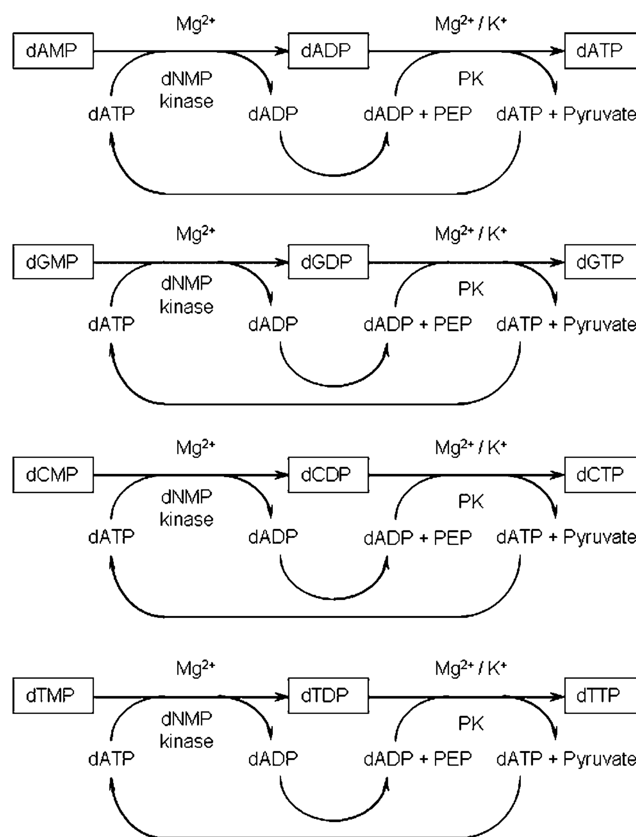

Figure 1. Coupled reaction scheme for the total biosynthesis of dNTP from dNMP.

using PK as a nonspecific deoxynucleoside diphosphate kinase enzyme. The reaction mechanism and kinetics have been studied and reported earlier (Bao and Ryu, 2005; Bao et al., 2005). Phosphoenolpyruvate (PEP) is used as the phosphate donor in the second reaction step.

The four genes encoding these deoxynucleoside monophosphate kinases (dNMP kinases) had been cloned into the *E. coli* and the enzymes were expressed using the recombinant *E. coli* strains as reported in our previous work (Bao and Ryu, 2006). The reaction mechanism and kinetics of these kinase enzymes have also been studied and reported (Bao and Ryu, 2006). We found that the phosphate donor substrates of the four enzymes are limited to only ATP and dATP. In this work, dATP is selected as the regeneration cofactor in the four coupled reactions for the total synthesis of the all dNTP components.

A potential application of unnatural nucleotide triphosphates derivatives could be of particular interest to pharmaceutical industry. The unnatural nucleoside or deoxynucleoside triphosphates (un-dNTP) can be used to produce unnatural deoxyribozymes with unique catalytic properties for potential medicinal application (Jäschke and Seelig, 2000; Dass, 2004). Preparation of un-dNTP from the unnatural deoxynucleoside monophosphates derivatives

DOI 10.1002/bit

(un-dNMP) is of some importance to the screening of the deoxyribozymes with diverse catalytic activities and potential application to medicine. To generate a large pool of un-dNMP, such chemical modifications of dNMP as functional group addition, replacement, and reconstruction have been successfully performed. However, there are only few examples of the modification of dNTP using the chemical method to generate a pool of un-dNTP, because dNTP possesses the high energy and high reactive phosphoryl bonds. The enzymatic method for the natural dNTP biosynthesis is of potential importance to preparation of a pool of the un-dNTP using the modified un-dNMP derivatives as the starting material.

The total enzymatic syntheses of the four dNTP components were carried out in four separate operations under the high substrate concentrations which is aimed at practical applications. The optimal conditions for individual reactions were studied and complete conversions of dNMP to dNTP have been demonstrated. The dNTP obtained from the reaction system was analyzed for their purity and quality. After a simple separation step, the dNTP products were obtained and tested for PCR application for gene amplification using varying sizes of genes from different microbial sources. The quality and purity of dNTP obtained from this work were compared to the dNTP from commercial sources and found to be equivalent for PCR application. More specifically, the goals of this work are to: (a) investigate the effects of reaction conditions including Mg^{2+}, K^{+}, and pH on the total biosynthesis of dNTP under the high substrate concentration for practical application; (b) optimize the reaction conditions of the total biosynthesis of dNTP; and (c) study the feasibility of practical PCR application of the dNTP produced by the method reported in this paper.

Materials and Methods

Materials

All chemicals and enzymes used in this study, including 2′-deoxyadenosine 5′-monophosphate sodium salt (dAMP), 2′-deoxyguanosine 5′-monophosphate sodium salt (dGMP), 2′-deoxycytidine 5′-monophosphate sodium salt (dCMP), 2′-deoxythymidine 5′-monophosphate sodium salt (dTMP), phosphoenolpyruvic acid monopotassium salt (PEP), pyruvate kinase from rabbit muscle (PK), L-lactate dehydrogenase from porcine heart muscle (LDH), lyticase, nicotinamide adenine dinucleotide (NADH-reduced form), and isopropyl-beta-D-thiogalactopyranoside (IPTG) were purchased from Sigma–Aldrich (St. Louis, MO). Lysozyme is from Roche (Indianapolis, IN). Tween 20 and Nonidet P40 were from MP Biomedicals (Irvine, CA). The LB medium used for *E. coli* cultivation contains 10 g peptone, 5 g yeast extract, 100 mg ampicillin, and 5 g sodium chloride per liter deionized water. To make the plates for solid surface culture, 20 g agar was added to one liter LB medium solution.

Organisms and Plasmids

The organisms used in this study include four recombinant *E. coli* BL21(DE3) strains, BL21(DE3)-JB1, BL21(DE3)-JB2, BL21(DE3)-JB3, and BL21(DE3)-JB4, for production of the four dNMP kinases used in the biosynthesis of dNDP. The details of these strains, plasmids, and the genes encoded for the dNMP kinases are shown in Table I. The recombinant strains were constructed and reported earlier (Bao and Ryu, 2006). The vector plasmid pET-17b and its host *E. coli* strain BL21(DE3) were purchased from Novagen (Madison, WI). The gene source was *Saccharomyces cerevisiae* ATCC 2601 strain which was purchased from ATCC.

Expression of the Kinase Enzymes

One liter fermentation medium was inoculated with 0.5 mL seed culture having $OD_{600} = 0.6$ cell concentration. The freshly transformed *E. coli* strain BL21(DE3) containing expression vectors were grown in LB medium containing 100 μg/mL of ampicillin for about 12 h. The culture temperature was 37°C for expressions of dAMP kinase, dGMP kinase, and dTMP kinase, and 25°C for dCMP kinase expression, respectively. The cells were harvested and washed with Buffer A (50 mM Tris-HCl, 50 mM dextrose, and 1 mM EDTA, pH 7.9). The washed cells were resuspended in 50 mL of pre-lysis buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 4 mg/mL lysozyme, pH 7.9) and

Table I. Recombinant strains for expression of deoxynucleoside monophosphate kinases (dNMP kinases).

Strains or host strains	BL21(DE3)-JB1	BL21(DE3)-JB2	BL21(DE3)-JB3	BL21(DE3)-JB4
Proteins to be expressed	dAMP kinase	dGMP kinase	dCMP kinase	dTMP kinase
Protein MW (Da)	24,255	20,637	22,933	24,687
Plasmids contained	pET17b-JB1	pET17b-JB2	pET17b-JB3	pET17b-JB4
Genes encoded	*ADK1*	*GUK1*	*URA6*	*CDC8*
Genes length (bp)	669	564	615	651
Names in SGD[a]	YDR226W	YDR454C	YKL024C	YJR057W
Primary SGDID[b]	S000002634	S000002862	S000001507	S000003818

[a]SGD: *Saccharomyces* Genome Database (http://www.yeastgenome.org/).
[b]SGDID: the identifying number of a specific gene within SGD.

incubated for 30 min at ambient temperature. 50 mL of lysis buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 1 mM PMSF, 0.5% Tween 20, 0.5% Nonidet P40, pH 7.9) was added and the cells were incubated for 30 min at room temperature and another 30 min at 37°C. The lysate mixture was centrifuged and 60 g of powdered ammonium sulfate was added to the supernatant obtained from 1-L whole broth harvested. The protein precipitate was recovered by centrifugation in 20 mL of Buffer A. While the enzyme was eluted with Buffer A from solution through a Sephadex G-100 column (80 cm × 1 cm), the protein was monitored by a UV detector at 280 nm and SDS-PAGE. All the SDS-PAGE protein samples were analyzed on 15% polyacrylamide gel and stained with Coomassie brilliant blue R-250.

To study the effect of pH on the reaction rate of all four dNTP components, 50 parts of Reagent solution A (2% sodium carbonate, 1% biocinchoninic acid, 0.16% sodium tartrate, 0.4% sodium hydroxide, and 0.95% sodium bicarbonate) and 1 part of Reagent solution B (4% $CuSO_4$) were mixed to make up the working solution for the protein assay. The assay tubes were incubated at 60°C for 30 min. The tubes were cooled to ambient temperature and the absorbance was read at 562 nm in 1 mL cuvette. The protein concentration was calibrated using BSA.

The Enzyme Activity Assay

The enzyme activity of the collected protein fractions was assayed using spectrophotometer at 340 nm. The assay was carried out at 30°C in a 0.5 mL quartz cuvette containing 50 mM Tris buffer solution (100 mM KCl, 4 mM $MgCl_2$, 10 U PK per mL, 10 U LDH per mL, 10 mg NADH per mL, 0.5 mM PEP, 1.0 mM dNMP, and 1.0 mM ATP at pH 8.0). Each reaction cell contained 10 μL of NADH (10 mg/mL), 10 μL of lactate dehydrogenase (LDH) solution (500 U/mL), 10 μl of pyruvate kinase solution (500 U/mL), and 25 μL PEP solution (10 mM). The reaction temperature was maintained at 30°C.

Reactions With High dNMP Substrate Concentration

The reactions with high dNMP substrate concentration and its enzymatic conversion to dNTP was carried out in a 5 mL glass test tube. The progress of reaction was analyzed using high performance liquid chromatography (HPLC, LC-9A, Shimadzu, Kyoto, Japan) with Intensil ODS-2 column (4.6 mm × 150 mm, GL Sciences, Tokyo, Japan). The reaction mixture was prepared in 0.5 mL of 50 mM Tris buffer solution by adding predetermined amount of dNMP, PEP, potassium chloride, and magnesium chloride. The reaction mixtures were incubated in a constant temperature water bath maintained at 30°C. Samples were assayed periodically during the reaction. The reaction was stopped by addition of hydrochloric acid to lower the pH below 3. The samples were filtrated through Diaflo Ultrafilter membrane YM10 with the molecular cut-off of 10,000 to remove soluble enzymes. The diluted samples were eluted by the solution containing 10 mM each of KH_2PO_4 and K_2HPO_4 at pH 7.0. The elution flow rate of the mobile phase was 1.0 mL/min at ambient temperature and the absorbance was monitored at 254 nm.

PCR Application of dNTP Produced for Gene Amplification

An Eppendorff Mastercycler machine (Westbury, NY) was used for PCR reactions. The oligonucleotide primers were designed using the Web Primer tool (http://seq.yeastgenome.org/cgi-bin/web-primer) and synthesized by Operon Technologies (Huntsville, AL). The primer sequences used are shown in Table II. The genes were amplified using Qiagen Taq PCR Core Kit (Valencia, CA). The dNTP product obtained from this work was used at the concentration of 0.2 mM in the PCR reaction solution. The dNTP product from Invitrogen was used as a reference for comparison purpose. The PCR mixture was prepared by mixing 5 μL of 10x Buffer, 5 μL of 10× dNTP (0.5 mM), 1 μL of forward and reverse primers (0.5 μM), 0.5 μL of *Taq* enzyme (2.5U), 2 μL of template (total 1μg), and add ultra pure water to total 50 μL. The conditions used for PCR reaction are: the initial denaturation at 94°C for 3 min, three step cycling for 25 cycles; denaturation for 30 s at 94°C, annealing for 1 min at 50°C, and extension for 2 min at 72°C, and the final extension for 10 min at 72°C.

The PCR reaction solutions for the dNTP heat stability test were prepared by mixing 5 μL of 10× buffer, 5 μL of 10× dNTP (0.5 mM) and bringing it to total 45 μL by adding ultra-pure water. Then the PCR cycle was carried out under the same PCR cycle conditions. After the cycle was over, 1 μL of forward and reverse primers (0.5 μM), 0.5 μL of *Taq* enzyme (2.5 U), 2 μL of template (total 1 μg) were added into the same PCR tubes containing 5 μL of 10× Buffer and 5 μL of 10× dNTP. The PCR cycle was carried out again using the same PCR cycle.

Results

Effects of Reaction Conditions on the Total Synthesis of dNTP: Mg^{2+} and K^+ ion Concentrations, pH, and Different Substrates

In this study the feasibility of high yield and high conversion of dNMP to dNTP was tested by using high substrate concentrations at the level of 100 mM of dNMP and 200–250 mM of PEP for practical application. The effects of Mg^{2+} and K^+ concentrations on the initial reaction rates of the total synthesis of all four dNTP were studied to find their optimal concentrations. Figure 2(a) shows that the reaction rates for the syntheses of four dNTP components increase with increasing Mg^{2+} concentration, go through maxima, and decrease thereafter.

DOI 10.1002/bit

Table II. Genes to be amplified using dNTP from Invitrogen, Promega, and the present work.

Gene 1	Source	*gfo* promoter
	Size	276 bp
	GenBank access	M97379 (regions 1–253)
	Forward primer	CGCGGATCC CAGAAATAATTATCTGACAGC
	Reverse primer	GCGATGTTAATCGTGTTCATAATCCTTGTTTCTTTCTTAA
	Template	*Zymomonas mobilis* ATCC 29191 (ZM6) genomic DNA
Gene 2	Source	*URA6*
	Size	615 bp
	GenBank access	NC_001143 (regions 392,169–392,783)
	Forward primer	CGGGATCCCATATGACAGCTGCCACTACATCAC
	Reverse primer	GGGCCGGAATTCCTATAAGCTATCACGGATAGCG TGTT
	Template	*Saccharomyces cerevisiae* ATCC 2601 genomic DNA
Gene 3	Source	*sucE3*
	Size	1,590 bp
	GenBank access	D17524 (regions 2,421–3,972)
	Forward primer	CGGGATCCATTCATTGTAAATGACGTTTC
	Reverse primer	CGGGATCCAAGCTTCGGGTTTTTCTTACT
	Template	*Zymomonas mobilis* ATCC 29191 (ZM6) genomic DNA
Gene 4	Source	*T7 Gene 1*
	Size	2,692 bp
	GenBank access	V01146 (regions 3,171–5,822)
	Forward primer	TTAAGAAAGAAACAAGGATTATGAACACGATTAACATCGC
	Reverse primer	AACAGGAAAAATAAGTCTGTTTACGCGAACGCGAAGTCCG
	Template	*E. coli* BL21(DE3) genomic DNA

Although Mg^{2+} concentrations corresponding to the maximum reaction rates vary slightly with different dNMP substrate used, the optimal Mg^{2+} concentration was found to be at about 100 mM, for production of all four dNTP. This Mg^{2+} concentration was found to be about the same order of magnitude as that of dNMP concentrations used. Figure 2(b) shows that the effect of K^+ concentration on the reaction rates for production of all four dNTP components are similar to that of Mg^{2+}. K^+ is required for activation of PK in the second phosphorylation step, from dNDP to dNTP. The reaction rates for all four dNTP components increase with increasing K^+ concentration, go through maxima, and slightly decline thereafter. The K^+ concentration of about 200 mM is found to be the optimal for production of all four dNTP components. This K^+ concentration was also found to be about the same order of magnitude as that of PEP concentration which is the phosphate donor used in the second phosphorylation reaction step.

The effect of pH on the phosphorylation reaction rate, for both the first reaction step from dNMP to dNDP and the second reaction step from dNDP to dNTP, were essentially the same (Bao and Ryu, 2005, 2006; Bao et al., 2005). The reaction rates of all four dNTP formation increase with

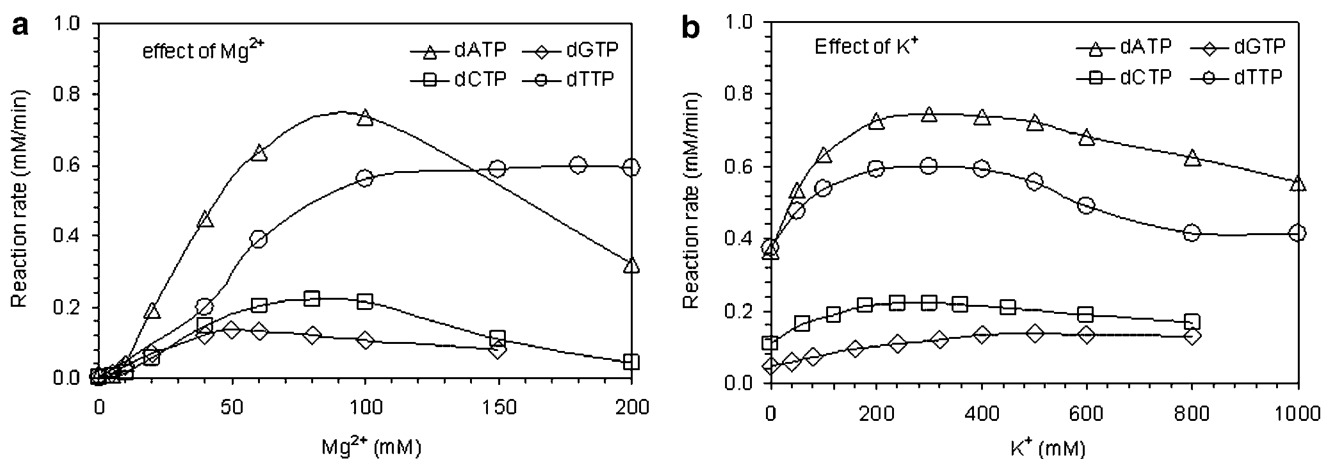

Figure 2. Effect of ion concentration on the total biosynthesis of dNTP at 30°C in 50 mM Tris with pH of 8.0. One hundred millimolar of dNMP, 100 mM ATP, 100 mM PEP, dNMP kinase 50 U/mL, PK 4.5 U/mL. The total protein concentration were 0.29, 0.57, 0.30, 0.29 mg/mL for the biosynthesis of dATP, dGTP, dCTP, and dTTP, respectively. **a**: 500 mM potassium chloride, 100 mM magnesium chloride. **b**: 100 mM magnesium chloride, 500 mM potassium chloride.

increasing pH, go through a maxima at pH 8.0, and declines thereafter. Thus, the optimal pH for the total synthesis of all four dNTP components was determined to be about 8.0.

The specific activities of kinase enzymes determined for the given substrates are shown in Table III. Among dNMP kinases, dAMP kinase shows the highest activity for dADP formation using either ATP or dATP as the regeneration cofactor. Though the kinase reaction rate with the use of dATP as the regeneration cofactor is only 26% of that with the use of ATP, it is still considerably higher by 7–14 times than that of other three dNMP kinases (Table III).

The reaction rates of dGMP kinase, dCMP kinase, and dTMP kinase for the formation of dGDP, dCDP, and dTDP, respectively, show only a moderate activity. Table III also shows that ATP was the best regeneration cofactor for these three reactions. The reaction rates of dGMP kinase and dTMP kinase, when dATP is used as the regeneration cofactor, are about the same order of magnitude as with the ATP as the regeneration cofactor. However, the kinase reaction rates are about one or two orders of magnitude smaller when the final product of the individual reactions (dGTP, dCTP, and dTTP) are used as the second substrate or regeneration cofactor as compared with that when ATP or dATP are used as regeneration cofactor. For dCMP kinase, the reaction rate using dATP as the regeneration cofactor was approximately one-third of that using ATP, while the reaction rate using dCTP as the regeneration cofactor was only about one fifth of that using dATP. The reaction rates of dGMP kinase and dTMP kinase using dGTP and dTTP as regeneration cofactors, respectively, are significantly smaller than that of dCMP kinase with dCTP as the regeneration cofactor. For the pyruvate kinase in the second phosphorylation step, from dNDP to dNTP, the reaction rates varied by 1–5 orders of magnitude when the substrates ADP, dADP, dGDP, dCDP, and dTDP were used.

High Yield and High Conversion Process for dNTP Production From dNMP

Complete conversion of dNMP to dNTP was achieved when dNMP kinase and PK enzyme were used in the same reactor (Bao and Ryu, 2006). For production of all four dNTP components, dATP is used as the phosphate donor in the first phosphorylation step from dNMP to dNDP and PEP is used as the phosphate donor in the second phosphorylation step from dNDP to dNTP. The dATP is used as the regeneration cofactor or the second substrate in the coupled reactions as shown in Figure 1. The time courses and the reaction conditions for all four dNTP components are shown in Figure 3a–d. The dNMP kinase enzyme unit is defined as the enzyme to convert 1 μmol each of dNMP and dATP to 1 μmol each of dNDP and dADP at 30°C and pH 8.0 in 1 min. The PK enzyme unit is also defined as the enzyme to convert 1 μmol each of dNDP and PEP to 1μmol each of dNTP and pyruvate at 30°C and pH 8.0 in 1 min. In all four reactions, complete conversion of dNMP to dNTP can be achieved within a few hours under the given reaction conditions.

Figure 3(a) shows that the conversion from dAMP to dATP was completed within 2 h under the given reaction conditions. The maximum dATP production rate was 1.24 mM/min. For production of dATP using dAMP kinase and PK enzyme, both enzymes showed high activity for the two-step sequential phosphorylation reactions. As shown in Table III, the reaction rate for dATP production using dAMP kinase and PK were several orders of magnitude greater than that of dGTP production using dGMP kinase

Table III. Enzyme activity of deoxynucleoside monophosphate kinases (dNMP kinases) and pyruvate kinase (PK) on different substrates.

Enzyme	Substrate 1	Substrate 2 or regeneration cofactor	Product 1	Product 2	Specific activity (μmol/min mg protein)
AK	dAMP	ATP	dADP	ADP	266.0 ± 18.6
	dAMP	dATP	dADP	dADP	70.6 ± 4.13
GK	dGMP	ATP	dGDP	ADP	11.0 ± 0.70
	dGMP	dATP	dGDP	dADP	11.0 ± 1.10
	dGMP	dGTP	dGDP	dGDP	0.138 ± 0.01
CK	dCMP	ATP	dCDP	ADP	14.43 ± 0.94
	dCMP	dATP	dCDP	dADP	5.66 ± 0.48
	dCMP	dCTP	dCDP	dCDP	1.06 ± 0.018
TK	dTMP	ATP	dTDP	ADP	14.44 ± 0.40
	dTMP	dATP	dTDP	dADP	10.48 ± 0.14
	dTMP	dTTP	dTDP	dTDP	0.144 ± 0.0069
PK	ADP	PEP	ADP	Pyruvate	6302.1 ± 157.4
	dADP	PEP	dATP	Pyruvate	496.7 ± 25.8
	dGDP	PEP	dGTP	Pyruvate	7.19 ± 0.17
	dCDP	PEP	dCTP	Pyruvate	0.068 ± 0.002
	dTDP	PEP	dTTP	Pyruvate	0.041 ± 0.002

The assay of AK, GK, CK, and TK were carried out at 30°C in 50 mM Tris with pH of 8.0. One hundred millimolar of potassium chloride and 2 mM of magnesium chloride. The assay of PK was carried out at the same conditions except the magnesium chloride was 15 mM. All substrate concentration were 1.0 mM.

DOI 10.1002/bit

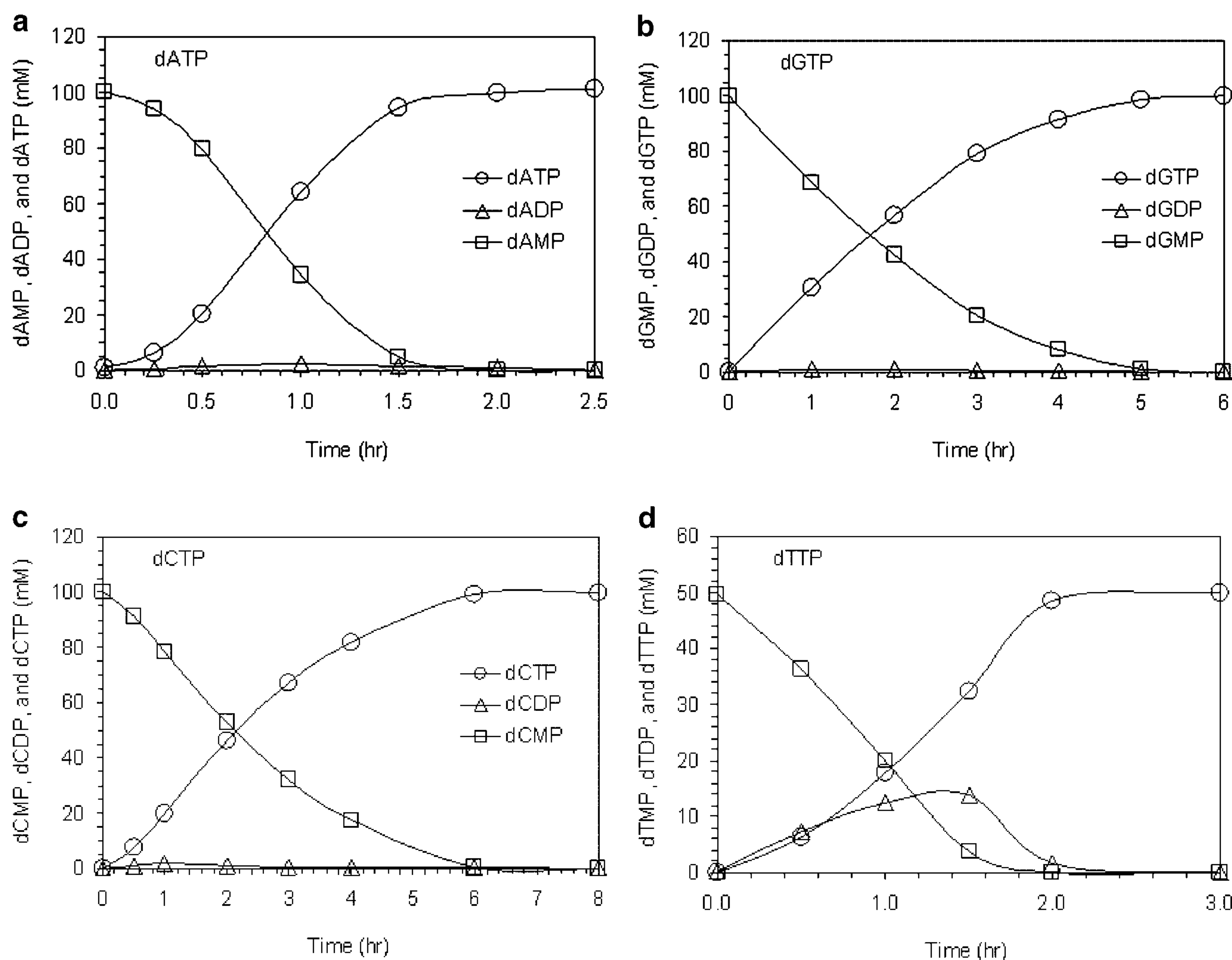

Figure 3. Time courses of the total biosynthesis of dNTP under high substrate concentration at 30°C in 50 mM Tris with pH of 8.0. The total protein concentration were 1.52, 1.55, 2.30, 1.50 mg/mL for the biosynthesis of dATP, dGTP, dCTP, and dTTP, respectively. **a**: 400 mM potassium chloride, and 100 mM magnesium chloride. 2.8 U/mL dAMP kinase, 5.5 U/mL PK, 100 mM dAMP, 250 mM PEP, 1.0 mM dATP. **b**: 200 mM potassium chloride, and 25 mM magnesium chloride. 25.5 U/mL dGMP kinase, 2.5 U/mL PK, 100 mM dGMP, 250 mM PEP, 1.0 mM dATP. **c**: 400 mM potassium chloride, and 100 mM magnesium chloride. 0.4 U/mL dCMP kinase, 0.15 U/mL PK, 100 mM dCMP, 250 mM PEP, 1.0 mM dATP. **d**: 200 mM potassium chloride, and 50 mM magnesium chloride. 2.0 U/mL dTMP kinase, 0.26 U/mL PK, 50 mM dTMP, 200 mM PEP, 1.0 mM dATP.

and PK, dCTP production using dCMP kinase and PK, dTTP production using dTMP kinase and PK, respectively. The dNDP intermediate product remained at a very low or zero concentration during the reaction, indicating that the ratio of enzymes added, dAMP kinase to PK, for the two sequential phosphorylation reactions were reasonably well balanced and neither enzyme was rate limiting.

Figure 3(b) shows that the conversion from dGMP to dGTP using dGMP kinase and PK was completed within 5.5 h under the given conditions. The maximum dGTP production rate was 0.452 mM/min. The result showing a very low concentration of dGDP intermediate product indicates that the second step phosphorylation from dGDP to dGTP by PK was not the rate limiting step. Table III reveals that the dGMP kinase activity for catalyzing dGMP to dGDP is only 15% of that for catalyzing dAMP to dADP using dAMP kinase when dATP was used as the regeneration cofactor or second substrate. The conversion reaction rate of dGMP to dGDP can be significantly increased by increasing the dGMP kinase and PK while maintaining a good balance between these enzymes and substrates.

Figure 3(c) shows that the conversion from dCMP to dCTP using dCMP kinase and PK was completed within 6.5 h under the given conditions. The dCTP production rate was 0.374 mM/min. The activity of dCMP kinase for catalyzing dCMP to dCDP was one order of magnitude smaller than that of dAMP kinase for catalyzing dAMP to dADP, but close to that of dGMP kinase for catalyzing dGMP to dGDP. On the other hand, the activity of PK for catalyzing dCDP to dCTP was considerably lower than that for catalyzing dADP to dATP (four orders of magnitude smaller) and dGDP to dGTP (two orders of magnitude

smaller). The conversion from dCDP to dCTP was enhanced by sufficient addition of PK in the high substrate concentration operation and the low level of dCDP indicated that the conversion of dCDP to dCTP was not the rate limiting step. The productivity of dCTP could be enhanced by increasing the dCMP kinase activity.

Figure 3(d) shows that the conversion of dTMP to dTTP was completed within 2.5 h under the given conditions. The maximum dTTP production rate was 0.404 mM/min. Table III shows that the activity of dTMP kinase for catalyzing dTMP to dTDP was smaller than that of dAMP kinase for catalyzing dAMP to dADP. The dTMP kinase activity is close to that of dGMP kinase that catalyzes dGMP to dGDP and about twice greater than that of dCMP kinase for catalyzing dCMP to dCDP. The relatively high concentration of the dTDP intermediate product and the relatively short conversion period of dTMP to dTTP indicate that the conversion of dTDP to dTTP could be the rate limiting step and the overall dTTP productivity could be improved by increasing the PK enzyme.

PCR Application Evaluation of dNTP Produced for Amplifying Genes From Different Sources

To test the quality of the dNTP product obtained from this work and compare with commercially available dNTP, HPLC analyses were carried out using the dNTP product obtained from Invitrogen (catalog number 10297-018, Carlsbad, CA) and Progema (catalog number U1240, Madison WI). Figure 4 indicates that the dNDP intermediate products have been converted to dNTP products completely and was absent in the final products obtained from this work. The purity and quality of dNTP products obtained from this work was found to be at least equivalent to or better than that found in the commercial dNTP product from Invitrogen and Promega.

The dNTP products obtained from the present method described in this paper have been tested for validation purpose by using them as the PCR reaction substrates and amplifying four genes of different lengths from different sources. The detailed information about the

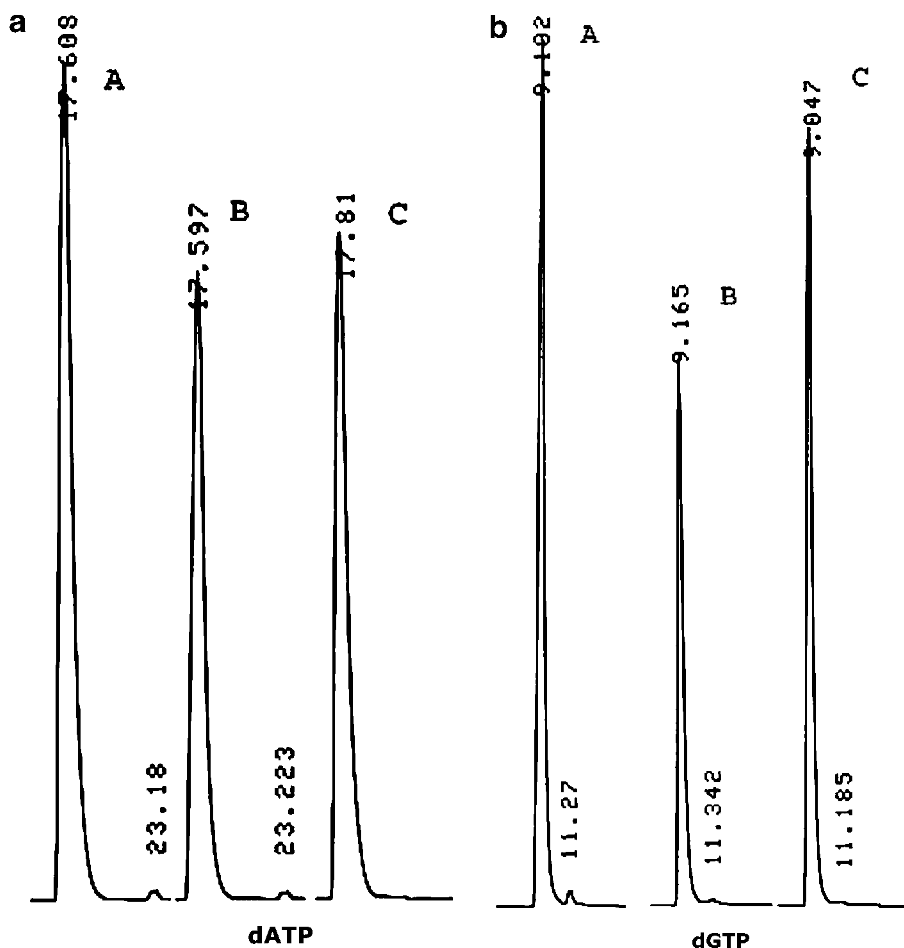

Figure 4. HPLC analysis of dNTP from different sources. **A**: Invitrogen dNTP product; **B**: Promega dNTP product; **C**: dNTP product of this work.

DOI 10.1002/bit

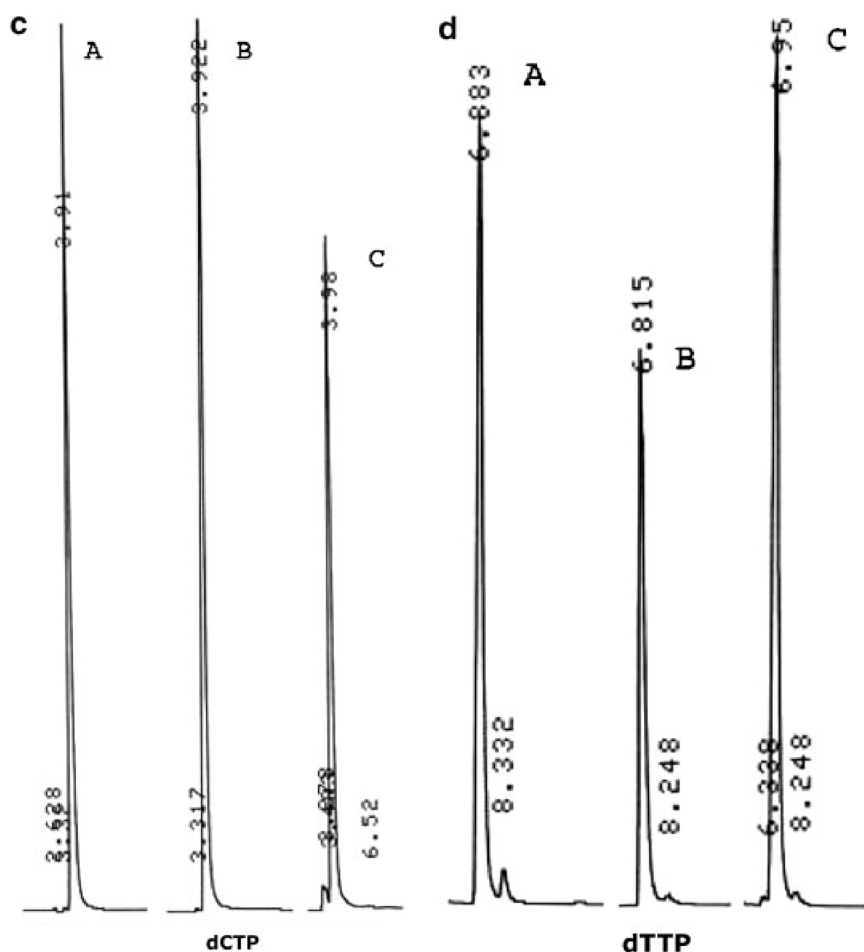

Figure 4. (*Continued*)

four genes used in our PCR application test, including gene sources, sizes, GenBank access number, the forward and reverse primers, and templates are given in Table II. The PCR test conditions were described in Materials and Method. The same amounts of the dNTP obtained from our present work and from Invitrogen were used. The results of DNA electrophoretic analyses of the PCR products obtained are shown in Figure 5. The results are identical and there was no noticeable difference between the PCR results using the dNTP obtained from this work and that from Invitrogen. These results suggest that the dNTP product obtained from this work can be validated for PCR application.

Figure 6 shows the heat stability of the dNTP product obtained from the present work and the commercial product from Invitrogen. The Gene 2 template was used as the target DNA. The results of electrophoretic analyses of PCR products show that there was no difference found in the PCR application when the dNTP reagent from this work was used and compared with those commercially available dNTP product.

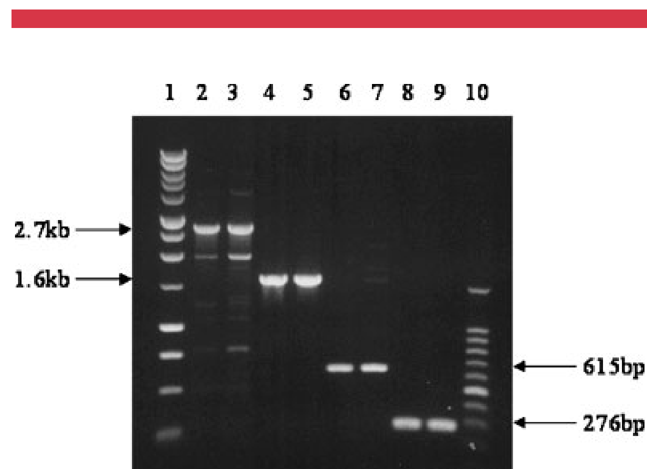

Figure 5. Gene amplification by PCR reactions using dNTP obtained from this work and the commercial products. 0.7% Agarose gel. 2 μL PCR product in each well. **lane 1**: 1 kb DNA maker; **lane 2**: Gene 4 using Invitrogen dNTP; **lane 3**: Gene 4 using dNTP from this work; **lane 4**: Gene 3 using Invitrogen dNTP; **lane 5**: Gene 3 using dNTP from this work; **lane 6**: Gene 2 using Invitrogen dNTP; **lane 7**: Gene 2 using dNTP from this work; **lane 8**: Gene 1 using Invitrogen dNTP; **lane 9**: Gene 1 using dNTP from this work; **lane 10**: 100 bp DNA marker.

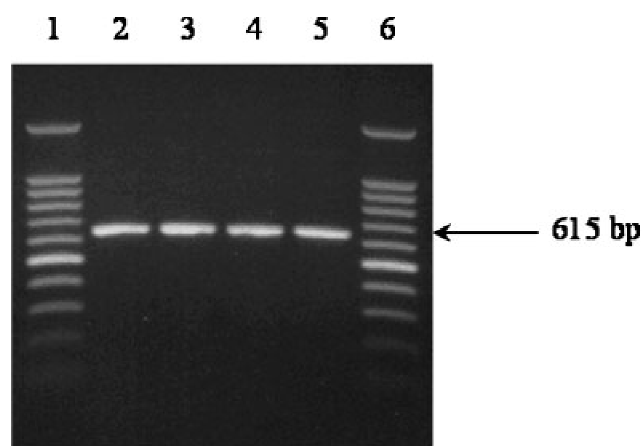

Figure 6. Heat stability of the dNTP product from the present work and commercial products. 0.7% Agarose gel. 2 μL PCR product in each well. **Lanes 1 and 6**, 100 bp DNA marker; **lane 2**, Gene 2 using Invitrogen dNTP without heat cycle; **lane 3**, Gene 2 using dNTP from this work without heat cycle; **lane 4**, Gene 2 using Invitrogen dNTP with heat cycle; **lane 5**, Gene 2 using dNTP from this work with heat cycle.

Discussion and Conclusions

The economically competitive and environmentally friendly bioprocess technology for production of dNTP using two-step enzymatic phosphorylation reactions in a single bioreactor has been developed and feasibility demonstrated. This technology offers significant advantages when compared to the chemical method currently in use. The first-step phosphorylation reaction from dNMP to dNDP, is catalyzed by four different dNMP kinases, namely, dAMP kinase, dGMP kinase, dCMP kinase, and dTMP kinase. Among the four enzymes required, only dAMP kinase obtained from animal tissues is commercially available to date (Sigma–Aldrich Biochemical and Reagents, 2004/2005). The other three enzymes, dGMP kinase, dCMP kinase, and dTMP kinase are not commercially available at the time of this reporting. In our work reported earlier, the four genes from *S. cerevisiae* strain encoding these four deoxynucleoside monophosphate kinase enzymes are cloned into the *E. coli* and the enzymes over-expressed from the recombinant *E. coli* strains (Bao and Ryu, 2006). These enzymes are produced at high expression levels and used for the biosynthesis of dNDP from dNMP. The second phosphorylation reaction step to dNTP from dNDP was also studied and reported earlier (Bao and Ryu, 2005, 2006; Bao et al., 2005).

In this study, the total biosynthesis of all dNTP components starting from dNMP to dNDP in the first step and to dNTP in the second step all in a single bioreactor system has been developed and demonstrated. The substrate concentrations at 100 mM of dNMP and 200–250 mM of PEP were used in the total synthesis of dNTP for the purpose of developing a practical technology. The substrate concentration and dNTP productivity are approximately 10-fold greater than that reported previously (Bao and Ryu, 2005, 2006; Bao et al., 2005). The optimal concentrations of magnesium and potassium ions corresponding to the high substrate concentrations were also examined. The results show that the optimal Mg^{2+} and K^{+} concentrations are found to be of the same order of magnitude as that of dNMP and PEP, respectively. The optimal pH was found to be the same for both the high and low substrate concentrations.

As shown in Figure 1, the reaction scheme requires four coupled reactions to accomplish a high overall conversion of dNMP to dNTP in a single bioreactor system. In each coupled reaction, a nucleoside triphosphate (NTP) or deoxynucleoside triphosphates (dNTP) can be used as a regeneration cofactor or the second substrate. The NTP or dNTP acts as a phosphate donor in the first phosphorylation reaction step to produce NDP or dNDP intermediate, then the NDP or dNDP acts as a phosphate acceptor to regenerate NTP or dNTP again in the second phosphorylation reaction step. Although all dNMP kinases showed a higher activity when ATP is used as the regeneration cofactor, the separation of dNTP from ATP in the final product mixture is more costly and difficult. Therefore, the final dNTP products in all four coupled reactions were selected as the best regeneration cofactor to be used in this process design. For example, the regeneration cofactors used are: dATP for dATP production, dGTP for dGTP production, dCTP for dCTP production, and dTTP for dTTP production, respectively. This process design strategy also reduces the purification cost for production of individual dNTP. In the case of dATP production, both the results of the enzyme activity assay shown in Table III and the results of high concentration operation shown in Figure 3 indicate that dATP is a good candidate as the regenerative cofactor. However, in the cases of production of dGTP, dCTP, and dTTP, as shown in Table III, the activity of dGMP kinase, dCMP kinase, and dTMP kinase using dGTP, dCTP, and dTTP as the regeneration cofactors, respectively, was significantly lower than that of dAMP kinase using dATP as the regenerative cofactor. On the other hand, dATP is a regeneration cofactor with a moderate activity for all four dNTP production. To keep the coupled reactions at a moderate reaction rate, a small portion of dATP was added to the coupled reactions for production of dATP, dGTP, dCTP, and dTTP (1 mM dATP in 100 mM dNMP) in a single bioreactor. Since dATP is one component in the dNTP mix for PCR application, it is not required to remove this small portion from the final dNTP product. Thus, purification was significantly simplified and there is no additional purification cost.

The enzyme assay result showed that the activity of PK highly depends on the substrates applied to the reactions. To balance the reaction rate in the coupled reactions, the addition of PK should be sufficient, especially in the cases of dCTP and dTTP productions.

DOI 10.1002/bit

This study has demonstrated the feasibility and established a totally integrated bioprocess technology towards the industrial application of the enzymatic production of dNTP. The results obtained from this study also suggest that the enzymatic method will bring about the significant economic and environmental benefits. Further studies are under way for improvement of: (1) dNMP production from low molecular weight DNA such as fish sperm or eggs and (2) dNTP productivity by manipulation of relative concentrations of enzymes, substrates, and cofactors based on the reaction kinetics in a single bioreactor system. The PEP regeneration method from pyruvate is also studied for further cost reduction. These future studies will further reduce the overall cost of dNTP production technology.

This research was supported by the National Science Foundation of China (Grant No. 20676037) and Shanghai Pujiang Program (Grant No. 06PJ14022), and also by the Industrial Gift Research Fund of Dewey Ryu at University of California, Davis, CA.

References

Bao J, Ryu DDY. 2005. Biosynthesis reaction mechanism and kinetics of deoxynucleoside triphosphates, dATP and dGTP. Biotechnol Bioeng 89:485–491.

Bao J, Ryu DDY. 2006. Cloning of deoxynucleoside monophosphate kinase genes and biosynthesis of deoxynucleoside diphosphates. Biotechnol Bioeng 93:572–580.

Bao J, Bruque G, Ryu Dewey DY. 2005. Biosynthesis of deoxynucleoside triphosphates, dCTP and dTTP: Reaction mechanism and kinetics. Enzyme Microb Technol 35:350–356.

Chambers R, Khorana H. 1957. Nucleoside polyphosphates. V. Syntheses of guanosine 5′-di- and triphosphates. J Am Chem Soc 79:3752–3756.

Chambers R, Moffatt J, Khorana H. 1957. Nucleoside polyphosphates. IV. A new synthesis of guanosine 5′-phosphate. J Am Chem Soc 79:3747–3753.

Dass CR. 2004. Deoxyribozymes: Cleaving a path to clinical trials. Trends Pharmacol Sci 25:395–397.

Environmental Protection Agency (EPA), Toxics Release Inventory. 2001. List of Toxic Chemicals. Pyridine, 110-86-1; DMF, 68-12-3.

Erlich H, Gelfand D, Sninsky J. 1991. Recent advances in the polymerase chain reaction. Science 252:1643–1651.

Jäschke A, Seelig B. 2000. Evolution of DNA and RNA as catalysts for chemical reactions. Curr Opin Chem Biol 4:257–262.

Ladner W, Whitesides G. 1985. Enzymatic synthesis of deoxyATP using DNA as starting material. J Org Chem 50:1076–1079.

Pavlov AR, Pavlova NV, Kozyavkin SA, Slesarev AI. 2004. Recent developments in the optimization of thermostable DNA polymerases for efficient applications. Trends Biotechnol 22:253–260.

Smith M, Khorana H. 1958. Nucleoside polyphosphates. VI. An improved and general method for the synthesis of ribo- and deoxyribonucleoside 5′-triphosphates. J Am Chem Soc 80:1141–1145.

Zhang K. 2003. China Patent CN1398977. Production process of deoxynucleoside triphosphate. International Classification: C12P19/38.